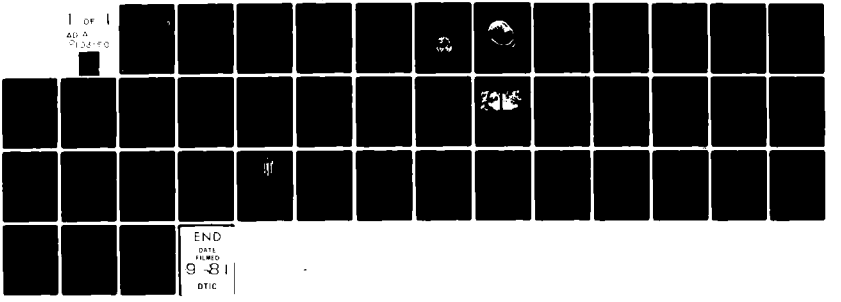
AD-A103 150 HARVARD MEDICAL SCHOOL BOSTON MA CHANNING LAB F/G 6/5
IMMUNOCHEMICAL INVESTIGATIONS OF CELL SURFACE ANTIGENS OF ANAER--ETC(U)
JAN 76 D L KASPER DAMD17-74-C-4056
UNCLASSIFIED NL



END
DATE FILMED
9-81
DTIC

2nd Annual Report

Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria

Annual Report

Dennis L. Kasper, M.D.

Jan. 15, 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

CONTRACT #DAMD17-74C-4056

Harvard Medical School
Channing Laboratory
Boston City Hospital
Boston, MA 02118

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A103 150	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria		5. TYPE OF REPORT & PERIOD COVERED Annual Interim report July 1, 1975-June 31, 1976
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Dennis L. Kasper, M.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD-17-74C-4056
9. PERFORMING ORGANIZATION NAME AND ADDRESS Harvard Medical School Channing Laboratory 774 Albany Street, Boston, MA 02118		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS M.C. Barstow, Jr. Director Office for Research Contracts, Holyoke Center Harvard University, Cambridge, MA 02138		12. REPORT DATE Jan 1976
		13. NUMBER OF PAGES 39
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) SAME		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

A- Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Not applicable

18. SUPPLEMENTARY NOTES

Not applicable

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Immunochemical	virulence	wound infections
Anaerobic bacteria	animal models	vaccines
capsules of bacteria	protection	
immunity	human infections	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The outer membrane of gram-negative bacteria is immunologically the most important structure of the cell because of its accessibility to host defense mechanisms. By electron micrographic studies, it has been shown that Bacteroides fragilis has a cell envelope typical of gram-negative bacteria. The outer membrane of this envelope was found to consist of protein, two polysaccharides bound to lipid in various degrees, and loosely bound lipid. The protein component of the outer membrane had a distinct pattern when

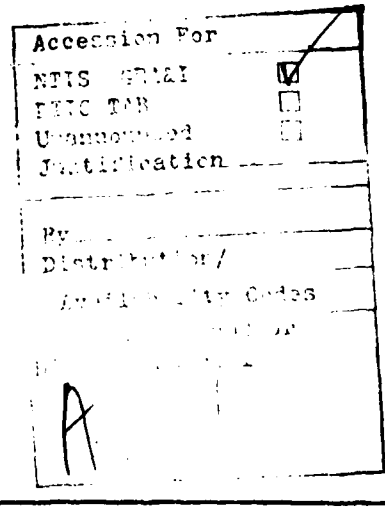
DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

41001

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

studied by polyacrylamide gel electrophoresis. Similar peptide band patterns were seen in all strains of Bacteroides fragilis subspecies fragilis studied, but this pattern was distinct from the pattern found in the outer membranes of other subspecies. The carbohydrates and the protein components were both associated with subspecies-specific immunoprecipitins. We propose that this antigenic complex represents the outer membrane of B. fragilis in its native state and that the immunologically important antigens of this organism are associated with this complex. A large molecular weight capsular polysaccharide has been isolated from strains of Bacteroides fragilis fragilis. By means of electron microscopy and staining with ruthenium red, the thick polysaccharide capsule has also been visulalized. With use of a radioactive antigen binding assay, antibody to this capsularpolysaccharide has been demonstrated in antisera prepared in rabbits to each of eight strains of B. fragilis fragilis. Antibody of similar specificity was not found in antisera prepared to Bacteroides melaninogenicus, to strains of B. fragilis vulgatus, B. fragilis distasonis and to only one of two strains of B. fragilis thetaiotaomicron. The radioactive antigen binding assay is a sensitive test for the detection of antibody to capsular polysaccharide. This polysaccharide antigen may form the basis of a serogrouping system for Bacteroides fragilis.

Bacterial lipopolysacchrides were extracted from Bacteroides fragilis subspecies fragilis. These lipopolysaccharides were found to lack 2-keto-3-desoxyoctonate and heptose, sugars which make up part of the inner core of most bacterial endotoxins. Over 98% of the lipid portion of this material could easily be removed with chloroform-methanol and alcohol, a finding which indicates a loose association between the polysaccharide and lipid moieties. The lipopolysaccharides caused gelation of the Limulus lysate at a concentration significantly higher than that for the endotoxin of Salmonella typhi. None of these extracts were found to be lethal in 10-day old chick embryos at doses of 200 ug per egg, wherease the endotoxin of Neisseria meningitidis was lethal at 1.2 ug per egg. The local Scwartzman reaction could not be induced at levels of up to 1,000 ug per rabbit, whereas a (control) endotoxin of Salmonella typhi induced this phenomenon at 3 ug per rabbit. Intact oxygen-killed B. fragilis failed to provoke the local Scwartzman reaction at doses of 2,500 ug. These results indicate that B. fragilis has a lipopolysaccharide different from that of most gram-negative bacteria. Although it retains some of the chemical and biologic properties of classical endotoxin, it seems to lack others. This observation may have significant slinical implications.



ANNUAL REPORT 1975-1976

UNITED STATES ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

CONTRACT #DAMD17-74C-4056

IMMUNOCHEMICAL INVESTIGATIONS OF CELL SURFACE ANTIGENS OF ANAEROBIC BACTERIA

Dennis L. Kasper, M.D.
Assistant Professor
of Medicine
Harvard Medical School
Channing Laboratory
Boston City Hospital

Annual Report

Resume of Accomplishments in the Past Year
(since the last Annual Report)

Bacteroides fragilis - Since the report of last year we have made much progress in our studies of two important determinants of this organism, the capsular polysaccharide and the endotoxin. The description of each of these investigations follows in the next two sections.

I. Capsule

A. Chemical and morphologic identification

Studies in our laboratory have shown that B. fragilis, an anaerobic, non-motile, gram-negative rod, has a cell wall structure similar to that of most aerobic gram-negative bacteria (1). In our investigations of the outer cell wall membrane complex of B. fragilis, the membrane was found to contain an important protein component which was shared by all studied strains of subspecies fragilis; similarly sized proteins were not found in representative strains of other subspecies. The outer cell wall membrane contained two major antigenic fractions which were separable by means of the disaggregation of one of these components with chromatography in an endotoxin depolymerizing buffer. This disaggregated antigenic fraction has been shown to be a lipopolysaccharide and to be immunochemically and biologically identical to the endotoxin of B. fragilis fragilis, as extracted by the classical phenol-water technique (2). The other antigenic fraction, eluted at the void volume of the column, had equal quantities of protein and carbohydrate. Both the void volume fraction and the endotoxin fraction had five sugars as demonstrated by paper chromatography. Four of these five sugars were shared by both major antigenic fractions. Chemical analysis of carbohydrates in both fractions revealed hexose, hexosamine, and methyl pentose in different molar ratios.

Previous immunologic studies had shown that the void volume material, when tested in immunodiffusion slides against antiserum prepared to subspecies fragilis, produced two immunoprecipitin lines. Trypsin treatment of this void volume material eliminated one of these immunoprecipitins.

Further characterization of the void volume carbohydrate by electron microscopy and immunochemistry have demonstrated that this material is a capsular polysaccharide.

1. Electron microscopy of strain 23745. Bacteroides fragilis ss. fragilis. This strain was grown in a 50 ml volume of a modified thioglycollate broth. Suspensions of whole bacteria were prefixed by the addition of 2.5% gluteraldehyde in 0.1 M Na cacodylate buffer to the broth; the final concentration of fixative was adjusted to 0.5%. $CaCl_2$ was added until a final concentration of 0.05% was achieved. After prefixation for 2 h at 4 C, the suspensions were again centrifuged at 12,000 g. The resulting

pellets were fixed for 2 h at 4 C in the buffered 2.5% gluteraldehyde, washed briefly with buffer, and fixed again for 60 min in aqueous 1% OsO_4.

The pellets were then exposed to 0.5% uranyl acetate in Michaelis buffer (pH 5.0) for 2 h at 24 C. One-mm blocks of the pellets were then dehydrated in graded alcohols and embedded in Epon 812 according to Luft (3). Thin sections were stained with lead citrate and examined with a Jem 100 B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

Polysaccharide capsules are not seen with routine staining techniques. So that bacterial capsules can be visualized, special stains such as ruthenium red are necessary. Samples prepared for staining with ruthenium red were treated in the exact manner described above, except that ruthenium red was added at a concentration of 700 parts per million to both the prefixation and fixation steps (4).

The cell envelope structure and morphology of B. fragilis were investigated by electron microscopy. In figure 1, untreated cells are seen after 36 h of growth in modified thioglycollate broth. These organisms exhibited a typical multiple layer cell envelope consisting of two trilaminar membranes and an electron dense peptidoglycan. Cytoplasmic membrane, peptidoglycan, and outer membrane are easily identified. The location of these layers was similar to the location of the same layers in aerobic gram-negative bacteria.

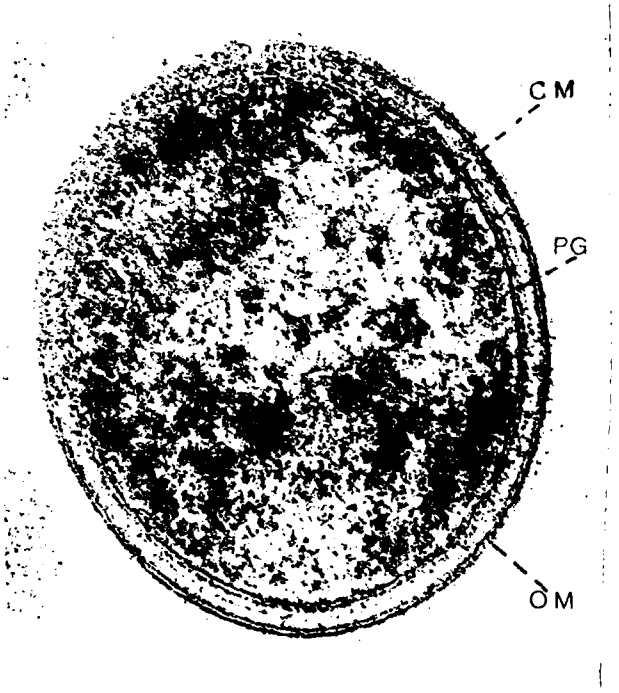


Figure 1: Electron micrograph of Bacteroides fragilis fragilis stained by standard techniques (X 120,000). OM=outer membrane; PG=peptidoglycan; CM= cytoplasmic membrane.

Organisms stained with ruthenium red are seen in figure 2. A thick capsule external to the outer membrane can be readily observed. Because of its staining with ruthenium red, this capsule can be identified as a polysaccharide. This darkly staining capsule is 1.5 to 2 times thicker than the cell wall itself. All organisms grown under these culture conditions consistently exhibit this feature.

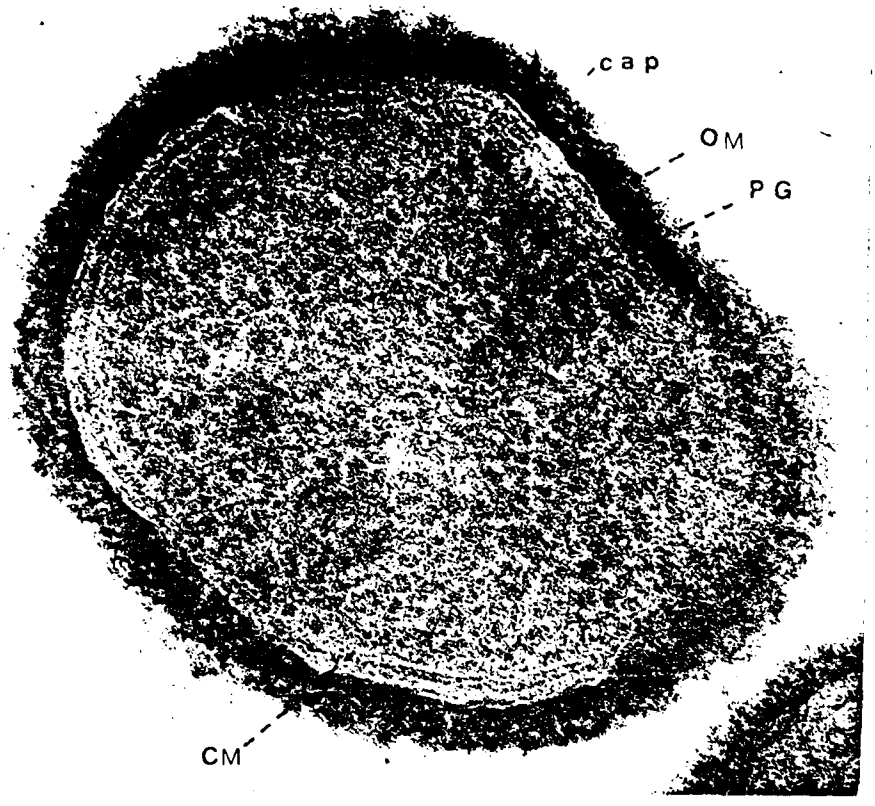


Figure 2. Electron micrograph of Bacteroides fragilis fragilis stained by means of ruthenium red so that polysaccharide capsule (cap) is visualized (X 120,000). OM=outer membrane; PG=peptidoglycan; CM=cytoplasmic membrane.

2. Immunochemical characterization

a. Initial fractionation of outer membrane

Outer membranes were prepared from strain 23745 (B. fragilis fragilis) grown in conditions identical to those previously described (4). Organisms were intrinsically labeled with 2.5 mCi (H^3) glucose per 1,200 ml of broth. The outer membrane purification procedure is detailed elsewhere (1).

The outer membrane was separated into two antigenic fractions with a technique previously described (1) by means of chromatography on a Sephadex G-100 column (Pharmacia, Uppsala, Sweden) equilibrated in an endotoxin disaggregating buffer. The void volume material (peak I) was found to contain equal quantities of protein and carbohydrate.

b. Radioactive antigen binding assay (RABA)

The procedure was similar to that dexcribed by Farr (5) as modified by Brandt (6). All sera tested were heat inactivated at 56 C for 30 min. Seventy microliters of undiluted serum, 125 µl of 0.1 M BBS, pH 8.45, and 5 µl of (H^3) labeled polysaccharide antigen were mixed in a glass test tube. The reaction mixture was incubated overnight, 16-18 h, at 4 C. After incubation, an equal volume (200 µl) of saturated ammonium sulfate (SAS) was added to precipitate the globulins, and the suspension was thoroughly mixed. The mixture was incubated for an additional 30 min, centrifuged for 30 min at 2,100 rpm at 24 C in a Universal Model UV International Centrifuge (International Equipment, Boston, Mass.) The precipitate was washed once with 1 ml 50% SAS. After washing, the suspension was again centrifuged and the precipitate was air dried for 1 h and dissolved in 1 ml Protosol (New England Nuclear). The solubilized pellet was then washed into a scintillation vial with 10 ml Liquefluor (New England Nuclear) in toluene 4.2% V:V. The samples were counted the next day in a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Silver Spring, Md).

A control for each experiment with radioactive antigen consisted of 70 µl normal rabbit serum and 130 µl BBS. After overnight incubation at 4 C, the control was fractionated with SAS as described above. After solubilizing the pellet in 1 ml Protosol, radiolabeled polysaccharide antigen added and the mixture was washed into a counting vial with 10 ml scintillation fluid.

The percentage of antigen bound for each test serum was computed from the equation:

$$\% \text{ antigen bound} = \frac{\text{test serum (cpm)}^* - \text{background (cpm)}}{\text{antigen control (cpm)} - \text{background (cpm)}} \times 100$$

*cpm = counts per min.

3. Initial experiments with the protein-polysaccharide complex as antigen

The molecular size of the protein-polysaccharide complex (Peak I) was estimated to be $>7.5 \times 10^6$ daltons because the fraction eluted in the void volume of the Sepharose 4B column (fig. 3).

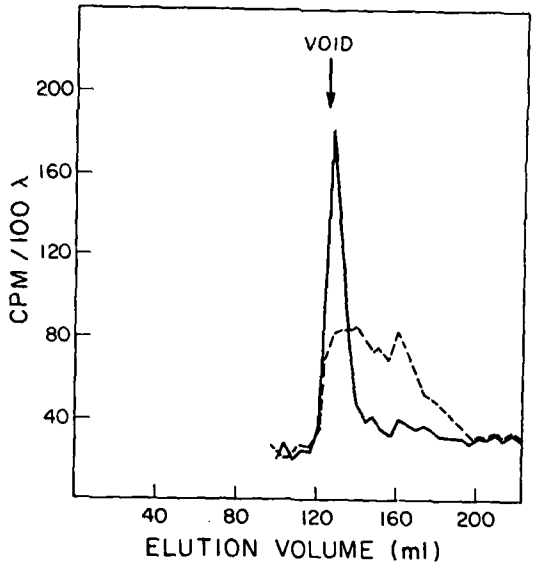


Figure 3. Elution profile of H^3 labeled polysaccharide (-----) and protein-polysaccharide (————) on Sepharose 4B. Void=elution volume of blue dextran; CPM=counts per min. of 100-sample.

In prior experiments (1) the complex was tested against antiserum to viable B. fragilis fragilis (prepared in rabbits); immunodiffusion slides produced two immunoprecipitins, indicating the immunological reactivity of the complex. Proof of this reactivity led to our development of the RABA for the detection of antibody to the antigen complex. This antigen was soluble in 50% SAS, a concentration at which serum globulins precipitate. The protein-polysaccharide complex, at increasing concentrations (indicated at μg protein, fig. 4) was tested in the RABA with hyperimmune rabbit antiserum prepared to strain 23745, B. fragilis fragilis.

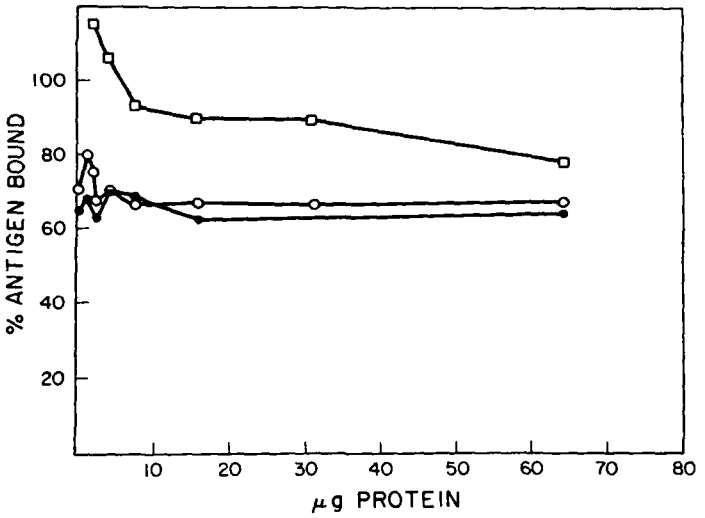


Figure 4. Percentage of antigen (protein-polysaccharide) bound by rabbit antisera at various concentrations of antigen protein. (□-□-□-□)=immune rabbit antiserum prepared to strain 23745 B. fragilis fragilis; (O-O-O)= normal rabbit serum; (●-●-●)=normal rabbit serum absorbed with strain 23745 B. fragilis fragilis.

This antiserum bound nearly all of the antigen added, even at high concentrations of antigen protein. However, at all antigen dilutions, there was a high percentage of antigen bound by the normal rabbit serum as well. If the mechanism involved were specific antigen-antibody interaction, incubation of antigen with normal serum would have shown a decrease in percent antigen bound over the midrange in this figure (30% - 80%). However, the percent binding at various antigen concentrations was relatively constant, indicating nonspecific binding. After extensive absorption of the normal rabbit serum with live homologous organisms, there was no apparent change in percentage

binding. We assumed, therefore, that the interaction between the antigen and the globulins in the serum was non-specific.

4. Purification of the polysaccharide capsule

Assuming that the non-specific binding was more likely the result of antigen protein-serum protein interaction than antigen polysaccharide-serum protein interaction, we removed the protein from the peak I antigen by means of trypsinization.

Peak I was dissolved in Tris-HCl buffer, pH 7.6, and when appropriate, 1 mg trypsin (Worthington Chemicals, Freehold, N.J.) per ml of antigen was added. The solution was incubated overnight at 37 C. The next day, an equal amount of trypsin was added and the solution was incubated for an additional 3-4 h. The polysaccharide capsular antigen was then purified on a 2.6- x 80-cm Sepharose 4B (Pharmacia) column equilibrated in Tris-HCl buffer, pH 7.6. Four-ml fractions were collected and 100\ of each fraction was counted for radioactivity with use of a scintillation fluid containing 4 g/l Omnifluor (New England Nuclear, Boston, Mass.) in a solution composed of toluene and ethylene glycol monomethyl ether (60:40, V:V). The column effluent was monitored at 280 nm by means of a UV monitor (Pharmacia) in tangent with the column. Degraded protein and trypsin showed absorption in fractions 93-111 (372-444 ml effluent).

Fractions 25-52 (100-208 ml effluent) were combined, concentration to 10 ml on a PM-30 ultrafiltration membrane (Amicon Corp., Lexington, Mass.) and precipitated with four volumes of cold absolute alcohol. The pellet was dissolved in water, lyophilized and the dry weight was determined. Fifty percent of the dry weight was accountable as hexose, as determined by the method of Roe (7) and less than 1% protein (8). The lyophilized pellet was dissolved in borate buffered saline (BBS) pH 8.4 and the specific activity was 214 cpm/µg.

Demonstrated in figure 3 is the elution profile of the peak I antigen before trypsin treatment and the pure polysaccharide after treatment. The polysaccharide portion has a molecular size of ~300,000 daltons. Chemically, this antigen was composed of approximately 50% hexose, 25% hexosamine, and 5% methyl pentose. By means of paper chromatography, we have identified sialic acid as the remaining constituent.

5. RABA with polysaccharide

The RABA was repeated (fig. 5) with the same antisera used in the initial experiment. With use of the polysaccharide antigen at various concentrations (indicated by µg hexose), marked differences were found between binding of antigen by unabsorbed normal rabbit serum and binding by the immune rabbit serum.

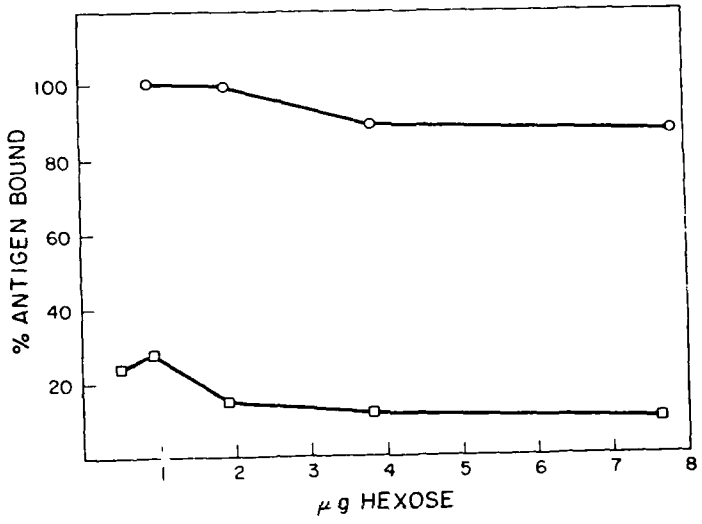


Figure 5. Percentage antigen (polysaccharide) bound at various concentrations of antigen hexose by immune rabbit antiserum (O-O-O) prepared to strain 23745, B. fragilis fragilis, and by normal rabbit serum (□-□-□).

Selection of antigen concentration.

For a determination of the relation between antigen concentration and antibody binding, the polysaccharide antigen was titrated against the sera of several individuals with varying quantities of natural antibody to this antigen. The sera of 20 laboratory volunteers were screened at an antigen concentration of 1.75 μg hexose in the RABA to identify individuals with various antibody levels. From this group, four individuals with midrange binding

were identified. The sera of these four individuals were titrated against antigen in concentrations decreasing to a level of 0.35 µg hexose. Predictable increases in binding are seen (figure 6) as antigen concentration is decreased. Also indicated here is the immune rabbit serum with its very high degree of binding. For further testing, a hexose concentration of 0.7 µg was thought to represent a reasonable midrange antigen concentration because of the linear relation between antigen binding and antigen concentration between 30% and 70% binding.

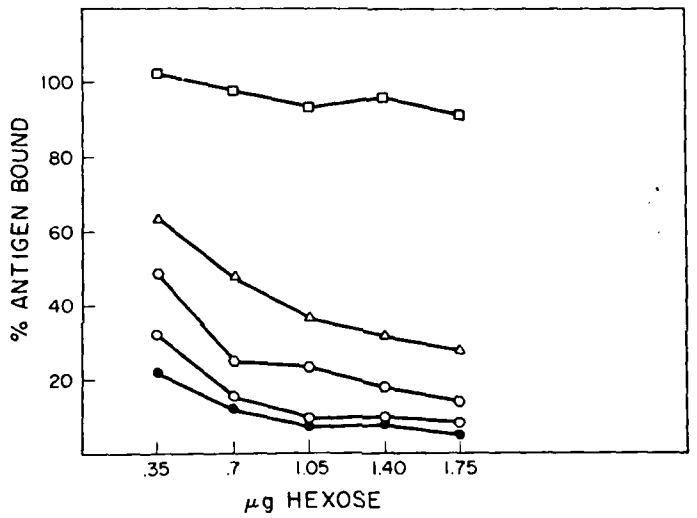


Figure 6. Percentage antigen (polysaccharide) bound at various concentrations of antigen hexose by various sera: (□-□-□-□)=immune rabbit antiserum prepared to strain 23745, B. fragilis fragilis; (△-△-△)=serum A-34 from human volunteer; (○-○-○)=serum A-13 from human volunteer; (O-O-O)=serum A-20 from human volunteer; (●-●-●)=serum A-39 from human volunteer.

6. Occurrence of antibody to the capsular polysaccharide in antisera to other strains of Bacteroides.

Antisera were prepared to viable organisms of various subspecies of B. fragilis and to one strain of B. melaninogenicus. Preimmunization and postimmunization sera were tested against the (H^3)-labeled polysaccharide antigen isolated from strain 23745 in the RABA. As can be seen in table 1, these immunizing organisms were obtained from various sources at wide geographic locales. The preimmunization sera all had very low levels of binding in this assay system. In all eight strains of subspecies fragilis, significant increases in the percentage of binding were seen with immune sera over preimmune sera. The percent of antigen bound varied between immunizing strains indicating significant degrees of antigenic similarity, perhaps either in composition or quantity of antigen present on the immunizing organism. Neither of the antisera to subspecies vulgatus showed a change in binding with the immune sera. However, the antiserum to one of two strains of subspecies thetaiotaomicron showed a significant change in binding. Antisera to single strains of B. fragilis distasonis and to B. melaninogenicus showed no rise in titer.

All antisera were tested in duplicate for a standard deviation of 1.7% binding.

Table 1

Radioactive Antigen Binding Assay Analysis of Rabbit Antisera to B. fragilis using Strain 23745 Peak 1 Polysaccharide as Antigen

Strain no.	Subspecies	Source	Prebleed	Hyperimmune
23745	fragilis	ATCC	10.3%	91.3%
2429	fragilis	Wadsworth-VA Hosp.	14.7%	89.5%
1262	fragilis	Temple U.	16.0%	29.7%
536	B. melaninogenicus	Wadsworth-VA	15.1%	13.3%
2242	vulgatus	Wadsworth-VA	10.2%	12.3%
2244	fragilis	Wadsworth-VA	6.0%	29.9%
2442	fragilis	Wadsworth-VA	11.1%	67.4%
8503	distasonis	ATCC	12.2%	15.4%
8482	vulgatus	ATCC	16.2%	16.6%
12290	thetaiotaomicron	ATCC	16.1%	42.0%
26877	fragilis	Boston City Hosp.	12.5%	63.4%
26783	fragilis	Boston City Hosp.	12.5%	29.3%
38310	fragilis	Boston City Hosp.	13.1%	65.0%

This data would indicate that strains of Bacteroides fragilis fragilis have a polysaccharide antigen which is shared by all strains tested of that subspecies. The association of this antigen with the outer membrane (1), its large molecular size, and the demonstration by electron microscopy of encapsulation would all indicate that the antigen described is a capsular polysaccharide.

With the RABA, we have found antibody to this capsular antigen in antisera prepared in rabbits to viable organisms of other strains of B. fragilis fragilis. Similar antibody was not found in antisera prepared to B. melaninogenicus, to strains of B. fragilis subspecies vulgatus, distasonis, and to only one of two strains of subspecies of thetaiotaomicron. Within subspecies fragilis, antisera to several strains showed variance in the amount of binding of the capsular polysaccharide of strain 23745 with immune sera. Although this variance may be due to antigenic heterogeneity, it may also be due to quantitative amounts of antigen present on the bacterial surface. Danielsson, et al. (9) have demonstrated some antigenic variance between strains of B. fragilis fragilis, suggesting the possibility of more than one serotype within this subspecies. Whereas our data do not completely refute the possibility of more than one serogroup within the subspecies fragilis, our methodology is more specific than that of the other report, in that the antigen we used was completely defined. The greater sensitivity of RABA for detecting antibody is evidenced in our prior inability to detect antibody to subspecies thetaiotaomicron strain 12290 when the Ouchterlony immunodiffusion analysis was used on the antisera.

With many bacteria, serogrouping is based on capsular polysaccharide antigens. Serogrouping has led to much valuable epidemiologic information regarding bacterial diseases. It would certainly seem that this capsular polysaccharide forms the basis of a serogrouping system within B. fragilis. However, these serogroups would incompletely conform to classification by subspecies, as was demonstrated by the induction of antibody to the thetaiotaomicron strain (12290). It is remarkable that not only is there great similarity among the polysaccharide capsules in strains of B. fragilis fragilis, but also there exists notably similar protein antigens associated with outer membranes of different strains (1). Similarity of outer membrane proteins within a biochemical group is in itself rather unusual. This observation and the finding of similar polysaccharides within this group are together, extraordinary.

Rowley (10) has suggested that perhaps the thickness of the capsule of a bacterium is a major factor in its virulence. Studies of Streptococcus pneumoniae have indicated that virulence varies with the quantitative amount of polysaccharide synthesized by different strains of the same type; these data show that one of the factors concerned with the mouse virulence of these strains is probably the genetic apparatus that controls the amount of polysaccharide produced (11). It is apparent from these studies that B. fragilis has a rather thick capsular polysaccharide associated with its outer membrane. With certain organisms, particularly S. pneumoniae, it has been found that although the specific capsular polysaccharide is nontoxic in the isolated state, it contributes to the virulence of the pneumococcus by acting as an anti-phagocytic agent. In a paper by Casciato, et al. (12), strains of B. fragilis fragilis were shown to be relatively resistant to serum bactericidal activity. Muschel (13) has indicated that the presence of a capsular polysaccharide may account for the resistance of an organism to the bactericidal activity of the complement-antibody system. The existence of a capsule in this bacterium may be a factor in its apparent virulence.

B. Further electron micrographic studies to determine the extent of encapsulation of strains of Bacteroides fragilis

Fifteen strains of B. fragilis have been studied by electron micro-

scopy for capsules by staining with ruthenium red as described in Section A. These strains represent the various subspecies of B. fragilis and were isolated from various clinical sites.

Table 2

Strain	Subspecies	Isolated from	Capsule
23745	fragilis	pleural fluid	++
2429	fragilis	blood	++
26783	fragilis	wound	+
8503	distasonis	feces	-
39675	distasonis	peritoneal pus	-
49256	distasonis	blood	-
530	distasonis		+/-
12290	thetaiotaomicron	wound	-
73996	thetaiotaomicron	wound	-
40575	thetaiotaomicron	blood	-
8482	vulgatus	feces	+ (20%) - (80%)
29960	vulgatus	placenta	-
2242	vulgatus	osteomyelitis leg drainage	-

Encapsulation has been found in all strains of subspecies fragilis studied. The EM data would indicate that few strains of other subspecies are encapsulated, even when isolated from blood cultures.

Using an indirect fluorescent antibody (IFA) test, which employs broth grown organisms, antisera prepared in rabbits to the capsular polysaccharide of B. fragilis fragilis and fluorescein conjugated goat anti-rabbit globulin, we have found that all of the strains of B. fragilis fragilis studied so far have specific fluorescence with the anti-capsular serum, whereas none of the strains of the other subspecies fluoresce. This has only been done on a limited number of strains (18 to date) but offers promise for a practical means of identifying B. fragilis fragilis in a clinical laboratory.

C. Virulence of encapsulated strains

In order to begin to ascribe this capsule a role as a virulence factor, we felt it important to know the frequency of isolations of the various subspecies from clinical specimens. Over a one and one half year period, we have been collecting all isolates of B. fragilis at Boston City Hospital, as well as serum, when available, of patients from whom this organism has been isolated. There are no similar published series on the frequency of isolation of the various subspecies of this organism (Table 3). As can be seen, subspecies fragilis was the most common isolate from blood, wound, or peritoneal infections. This must be considered in light of the fact that subspecies distasonis and vulgatus are the most common isolates from normal flora. We feel that this is highly suggestive of greater pathogenicity of this subspecies.

To further test this hypothesis, we have been collaborating with Drs. John Bartlett and Andrew Onderdonk in an experimental animal model of intraabdominal sepsis and peritonitis.

Table 3

Frequency of Isolation of Subspecies of Bacteroides fragilis from Various Clinical Sites*

Source	Subspecies									
	fragilis		vulgatus		distasonis		TIO		Overall	
wound	23	(70%)	1	(3%)	3	(9%)	6	(18%)	53/70	(47%)
peritoneal	6	(38%)	2	(13%)	5	(31%)	3	(19%)	16/70	(23%)
blood	10	(71%)	1	(7%)	2	(14%)	1	(7%)	14/70	(20%)
placenta	3	(43%)	4	(51%)	0				7/70	(10%)

* Patients collected from Boston City Hospital from September 1974 to November 1975.

D. The Model

Animals. Male Wistar rats (Simonsen Laboratories, Palo Alta, Ca.) weighing 160-180 grams are used in this study.

Inoculum. It is necessary to prepared the surgical implants to provide a uniform inoculum of microorganisms so that all animals receive an identical challenge. Such an inoculum is made by pooling the large bowels of 15 meat-fed rats in the following manner: The abdomen of each rat is aseptically opened. The cecum and distal large intestine are then clamped, excised, and immediately entered into an anaerobic glovebox (14). Intestinal contents are carefully extruded into a sterile beaker and the tissue in mascerated. An equal volume of pre-reduced peptone-yeast-glucose broth (PYG) is added to this material and vigorously mixed. The resultant slurry is filtered through two layers of surgical gauze into a second sterile beaker in order to remove large particulate matter and tissue. Ten percent weight/volume sterile barium sulfate is added and the inoculum is then divided into small aliquots (approximately 5 ml) which are placed in glass vials fitted with rubber stoppers and screw caps. The closed vials are removed from the chamber, immediately immersed in liquid nitrogen for four minutes and stored at -40 C until used.

Quantitative bacteriology has been performed on the quick frozen inoculum (15) by preparing serial 100-fold dilutions in the anaerobic chamber for plating on appropriate aerobic and anaerobic media. After incubation, colony types were enumerated, isolated and identified. This analysis revealed a total of 22 bacterial species including 13 anaerobes and nine aerobes (Table 4).

In highest concentration were two species of Eubacterium which were present at levels of $10^{7.5}$/ml. These organisms outnumbered the most frequent

aerobe in the inoculum by more than two logs. The next most frequent organisms were an anaerobic pleomorphic Gram-negative bacillus and an anaerobic nonsporulating Gram-positive bacillus. These organisms did not fit conventional classification schemes and could not be speciated. Several Clostridia species, Bacteroides fragilis, peptococci and Fusobacterium varium were present in concentrations of 10^5-$10^{6.1}$/ml. Enterococcus and E. coli were the predominant aerobic isolates, occurring in concentrations of $10^{5.4}$ and $10^{5.2}$/ml respectively. Several other facultative isolates were also present including Lactobacillus, Micrococcus, Corynebacterium, Proteus, α-hemolytic streptococcus and Moraxella.

Thus, the inoculum contains a polymicrobial flora in which anaerobes outnumber aerobes by a factor of 100:1. Intestinal studies in man have shown similar bacterial populations in terms of the ratio of aerobes to anaerobes and the major bacterial species (16).

Table 4

Bacteriology of the Inoculum

Anaerobes	Log cfu/ml	Aerobes	Log cfu/ml
Eubacterium tenue	7.5	Enterococcus	5.4
Eubacterium aerofaciens	7.5	Escherichia coli	5.2
Pleomorphic Gram-negative rod	7.0	Lactobacillus sp.	5.0
Non-sporeforming Gram-positive rod	6.3	Micrococcus sp.	4.5
Clostridium perfringens	6.1	Corynebacterium sp.	4.4
Clostridium paraputrificum	6.0	α-hemolytic streptococcus	4.0
Clostridium species	6.0	Proteus mirabilis	4.0
Bacteroides fragilis	5.8	Proteus morganii	3.9
Peptococcus morbillorum	5.7	Moraxella sp.	3.1
Peptococcus micros	5.8		
Fusobacterium varium	5.2		
Clostridium sartagoformum	5.2		
Clostridium tyrobutyricum	5.0		

Bacteriological studies were also performed on an inoculum prepared from grain-fed rats. In this inoculum, however, aerobes actually outnumbered anaerobes and the major isolates were quite different from those in the human intestine (15). Consequently, the experimental studies are performed with the inoculum prepared from meat-fed rats. The pooled intestinal contents from 15 such animals provide sufficient implants for approximately 1,200 rats.

Implantation of inocula. The frozen inoculum obtained from meat-fed rats is thawed in the anaerobic chamber. One half ml is placed in a sterile gelatin capsule, removed from the chamber and immediately implanted into rats anesthetized by intraperitoneal injection of 0.15 ml of Sodium Nembutal

(50 mg/ml). The abdomen of each animal is shaved, cleaned twice with 1% iodine, and a 1.5 cm anterior midline incision made through the abdomen wall and peritoneum. The capsule is then inserted into the pelvic region of each rat. The incision is closed with three or four interrupted 3-0 silk sutures and the animals returned to separate cages.

Anatomical studies. Deaths which occur within four hours are ascribed to anesthesia or to surgery and these animals are eliminated from the study. (Our previous observations indicate that this acute postoperative mortality occurs in approximately 5% of animals). All animals are observed at eight hour intervals throughout the test period. Rats which survive 10 days are sacrificed at that time. Complete autopsies are performed on all animals. This includes a careful search of the abdominal cavity for evidence of peritonitis or abscesses. The criterion for peritonitis is a free-flowing peritoneal exudate; for intraabdominal abscess it is a localized purulent collection.

Bacteriological studies. Quantitative bacteriology is performed on specimens obtained from the infected site of randomly selected rats immediately following sacrifice. Specimens are obtained with a tuberculin syringe and passed immediately into the anaerobic chamber for processing. A 0.1 ml sample of purulent material is placed in 9.9 ml of pre-reduced VPI dilution salts for serial 100-fold dilutions. Aliquots of 0.1 ml of each dilution are then spread on both pre-reduced and aerobic plating media to five final dilutions of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}/ml. Anaerobic media include: pre-reduced brucella agar base containing 6% sheep's blood and 10 µg/ml menadione (BMB); BMB containing 100 µg/ml neomycin sulfate; and laked blood agar containing 75 µg/ml kanamycin and 7.5 µg/ml vancomycin. These three media are incubated at 37 C inside the anaerobic chamber and held for 3-5 days. The following media are employed for aerobic and facultative isolates: blood agar plates incubated with increased CO_2; MacConkey's agar and Pfizer Selective Enterococcus Agar. These plates are incubated at 37 C for 24-28 hours. After incubation, colony types are enumerated, isolated and identified. Anaerobic isolates are identified according to the procedures outlined by the VPI Anaerobe Laboratory Manual (17). Enterobacteriaceae and other aerobic isolates are identified by established procedures (18).

E. Previous Observations with This Model

1. Natural course of infection in untreated animals (15,19)

Initial studies concerned the sequential bacteriologic and pathologic changes which occurred after implantation of a fecal inoculum. Variables tested were 1) the inoculum prepared from meat versus grain-fed rats; 2) addition of blood to the inoculum; 3) female versus male recipients and 4) different weights of the animals. Only results with the techniques described above under "The Model" will be summarized since these satisfied our original goals:

a. Standardized inoculum so that all animals receive an identical challenge
b. Infection which simulates naturally occurring disease

in humans by both clinical and microbiological parameters

c. Reproducible results according to well defined objective criteria

Anatomical changes have been studied in 106 male 160-180 gm Wistar rats which received the inoculum from meat-fed rats. Three control groups consisted of animals implanted with an autoclaved (sterile) inoculum, $BaSO_4$ alone and $BaSO_4$ plus sterile inoculum. The animals were observed daily and all rats which succumbed were autopsied for gross anatomical studies. In order to define the natural history of this infection, surviving rats were randomly sacrificed for autopsy examination at intervals of 4-14 days (Figure 7).

Among the 106 animals which received the inoculum, seven died between eight and 16 hours, and 21 (19.8%) were dead before 24 hours. Autopsy of these animals revealed that the gelatin capsules began to dissolve shortly after insertion, but even at eight hours, the inoculum was usually still localized in the pelvis. Within 24 hours a suppurative infection and ileus developed, and 0.2-0.5cc of peritonitis fluid had accumulated. The fur appeared ruffled, and the animals were lethargic and cold. At 48 hours, peritoneal adhesions began to appear anteriorly and loosely attached collections of purulent material were noted. By three days, 41 (39%) of the animals had died, and, at 4 days, 43% (46/106) of the animals had expired. There were no further natural deaths after this time.

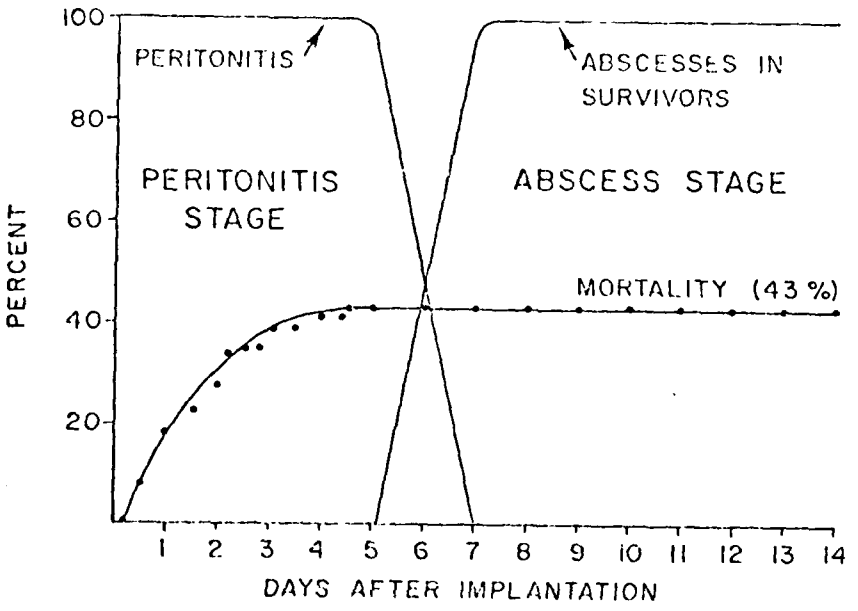


Figure 7: Biphasic disease course according to autopsy observations in 106 untreated Wistar rats sacrificed at various intervals after implantation. Mortality is expressed as cumulative percent (55).

By seven days a well-formed abscess was usually palpated inferiorly along the anterior abdominal wall. The surgical incision was well healed and peritoneal fluid was seldom present. All animals sacrificed at seven days or later had multiple localized purulent collections distributed throughout the abdominal cavity. Abscess cavities continued to enlarge and by two weeks contained 0.2-0.5 cc of pus (Figure 8).

Figure 8a: Typical intra-abdominal abscess in a rat sacrificed 10 days after implantation of the inoculum from meat-fed rats.

Figure 8b: Multiple large intraabdominal abscesses in rat sacrificed one month after implantation.

Control animals which received the sterile inoculum in gelatin capsules showed no signs of toxicity. At 14 days the animals were sacrificed and no pathologic changes could be found. The barium sulfate alone or the combination of $BaSO_4$ and sterile inoculum produced a similar clinical picture. At autopsy the only findings in these animals were multiple sterile granulomas.

Bacteriological studies of infected sites were performed on 20 animals. Samples were obtained at various intervals for up to two weeks following implantation. These specimens consistently yielded a complex mixed aerobic-anaerobic flora. There was a mean of 6.2 bacterial species/case including 3.2 aerobes and 3.0 anaerobes. Four organisms were uniformly present and numerically dominant: E. coli, enterococci, Bacteroides fragilis and Fusobacterium varium. Comparison of relative concentrations of these organisms

in peritonitis exudate and abscess contents showed that the two facultative species outranked the two anaerobes during the peritonitis stage, while the reverse applied to the abscess stage. According to rank order analysis, these differences are highly significant.

Blood cultures were obtained from randomly selected animals at 1-3 days, 7 days and 14 days. All ten animals sampled in the acute peritonitis stage had bacteremia, and many had polymicrobial bacteremia. Blood culture isolates in these ten animals were E. coli (9 rats), Proteus mirabilis (20), B. fragilis (21) and enterococci (22). At seven days bacteremia was less frequent and at 14 days the blood cultures failed to yield any pathogens.

These experiments show that the previously defined goals for an experimental model were satisfied. The fecal implant produced a predictable disease which followed a biphasic course. Initially, there was acute, often lethal, peritonitis. This was followed by the formation of intraabdominal abscesses at 5-7 days in all surviving animals. The pathologic changes and the organisms most frequently isolated from infective sites were similar to observations in human infections. Additionally, mortality and abscess were well defined objective criteria to judge different stages of the disease in future experiments.

Of particular interest were the sequential bacteriological changes which occurred during evolution of the disease. Starting with a complex inoculum containing at least 22 identifiable bacterial species, there was a simplification of this flora at the infected site. All specimens yielded a mixture of aerobes and anaerobes, but the relative concentrations of these two bacterial types changed during the course of the disease. Aerobes were predominant during initial peritonitis, a stage associated witn E. coli bacteremia and high mortality. Surviving animals uniformly developed localized intraabdominal abscesses in which the numerically dominant organisms were anaerobes, principally B. fragilis and F. varium.

In recent studies an additional approach has been used to further clarify the roles of specific bacteria in this experimental model. Instead of implanting a complex fecal inoculum, a challenge of selected bacterial strains singly and in various combinations was used. The organisms selected for these studies were those which dominated at infected sites in the initial studies using colonic contents: E. coli, enterococci, B. fragilis and F. varium.

There were 11 groups of 20 animals. Initially, all four organisms together were used, then each organism was implanted alone, and finally, we used all possible combinations of two species. The source of bacteria for these experiments was an intraabdominal abscess following the fecal implant. Broth cultures of each strain were diluted to achieve a final concentration of 1×10^8 cfu/ml. An exception was E. coli which was diluted to 5×10^7 cuf/ml. (This adjustment was necessary because preliminary experiments showed that the higher concentrations with E. coli were universally fatal.) The total volume of broth used in the inoculum was 0.5 ml in all animals. Thus, the total number of bacteria in the challenge was the same in all groups, with the exception of those receiving E. coli. In each experiment,

the challenge was prepared by mixing the broth culture, autoclaved colonic contents and $BaSO_4$ for insertion into the gelatin capsule. All surviving animals were sacrificed at 7 days for autopsy examination.

Table 5

Group	No. tested	Mortality		Abscesses in survivors	
(1) All four species	20	8/20	(40%)	12/12	(100%)
(2) Single species					
E. coli	20	13/20	(65%)	0/ 7	
Enterococci	20	0/20		0/20	
B. fragilis	20	0/20		0/20	
F. varium	20	0/20		0/20	
(3) Dual combinations					
E. coli + *B. fragilis*	20	7/19	(37%)	12/12	(100%)
E. coli + *F. varium*	19	6/19	(32%)	12/13	(92%)
Enterococcus + *B. fragilis*	20	0/20		19/20	(95%)
Enterococcus + *F. varium*	19	0/19		17/19	(89%)
Enterococcus + *E. coli*	20	5/20	(25%)	0/15	
B. fragilis + *F. varium*	19	0/19		1/20	(5%)

Results of these experiments are summarized in Table 5. The combination of all four species reproduced the previous observations using a complex fecal inoculum. Eight of the 20 animals expired by the 7th day, and all twelve survivors had intraabdominal abscess. Experiments in which each species was implanted separately showed mortality only in the group receiving *E. coli*, and none of the animals receiving a single species developed intraabdominal abscesses. These results suggested that microbial combinations were critical for abscess formation. The dual combination experiments again showed mortality only in the groups which received *E. coli* in the inoculum. Abscesses were largely restricted to experiments utilizing one facultative strain and one anaerobe. The incidence of abscesses was 89-100% in the groups which employed this type of combination. On the other hand, no abscesses were detected in animals receiving *E. coli* plus enterococci, and only one animal developed an abscess in those challenged with the rat isolate of *B. fragilis* and *F. varium*.

F. Recent Collaborative Investigations with Encapsulated Strains of B. fragilis

The strain of B. fragilis used in the experiments described above has been found by capsular staining techniques to be an unencapsulated strain of B. fragilis ss. other.

We next implanted the peritoneum of animals with strain 23745 ss. fragilis, the encapsulated subspecies described previously. These data are described in table 6. The encapsulated strain either by itself or in combination with the enterococcus induced abscess formation, while the unencapsulated strain (8503) only induced abscess formation in combination with the enterococcus, and not by itself.

Table 6

Initial Comparison of Encapsulated and Unencapsulated Strains of B. fragilis in Rat Model

# Rats	Inoculum & Strain #	Mortality	Abscess (%)	Culture
10	Entero, $5x10^7$ B. frag. ss. fragilis, 23745, $5x10^7$	0	80	6~B. frag. 6/6
10	Entero, $5x10^7$ B. frag. ss. distasonis, 8503, $5x10^7$	0	80	5 entero 3/5
10	B. frag. ss. fragilis 23745, $5x10^7$	0	90	5 B. frag. 4/5
10	B. frag. ss. distasonis 8503, $5x10^7$	0	20	2 B. frag. 1/2

Strain		
Encapsulated alone	90	4/5 (80)
Unencapsulated alone	20	1/2 (50)
Encapsulated + entero.	80	6/6 (100)
Unencapsulated + entero.	80	0/6 (0)

Table 7 summarized the results of a number of studies which showed that the encapsulated strains consistently caused abscess formation to a significantly greater degree than unencapsulated strains. Furthermore, heat killed encapsulated strains were sufficient to cause abscesses, either alone or in combination with unencapsulated strains. Killed encapsulated organisms induced sterile abscesses, which were not found in the control animals. In contrast, nearly all abscesses induced with live encapsulated strains yielded positive cultures.

Table 7

Summary of Recent Data on Rat Model

Group	Abscess	Abscess culture for B. fragilis
B. frag. ss. frag. (3 strains)*	19/20	9/10
B. frag. ss. dist. (2 strains)+	5/20	1/5
B. frag. ss. vulgatus (1 strain)+	3/10	0
B. frag. ss. TIO (1 strain)+	2/10	0
B. frag. ss. frag. (killed)*	8/10	0
B. frag. ss. frag. (killed)* B. frag. ss. dist. (live)+	17/20	0
B. frag. ss. other+ + Strep. sanguis	9/10	0
Sterile cecal contents (no $BaSO_4$)	0/10	0
Sterile broth (no cecal contents or $BaSO_4$)	0/10	0
Broth culture B. frag. ss. frag.* (no cecal contents or $BaSO_4$)	0/10	0
Broth culture B. frag. ss. dist.+ (no cecal contents or $BaSO_4$)	0/10	0

* encapsulated
\+ unencapsulated

We believe that these data stronly support the thesis, that encapsulated strains are more virulent than unencapsulated strains. Further studies will be described in the proposal which will allow proof that the capsule itself is the "virulence" factor.

G. Antibody Response in the Animal Model

To begin to evaluate the potential usefulness of the RABA in clinical infection, studies were done in several of the groups of animals described above, to detect antibody to the capsular material in experimental infection.

In Table 8, it is seen that animals fed meat or grain and observed for one month showed no changes in antibody levels. Animals receiving the enterococcus alone also showed no change in the level of circulating antibodies to the capsule of B. fragilis ss. fragilis. However, animals receiving B. fragilis

ss. fragilis alone or in combination with the enterococcus developed significant antibody rises as early as seven days after infection. Animals receiving killed ss. fragilis or live ss. fragilis without cecal contents or $BaSO_4$ similarly developed rises in antibody. Animals receiving strains of ss. distasonis, or vulgatus showed no rises in antibody, although animals receiving another strain of ss. fragilis or the fecal inoculum did show increased titers.

Table 8

Summary of Antibody Response in Rat Model to Capsule of ss. fragilis

Group (n)	Day after infection Serum Drawn	Reciprocal Mean Titer (50% binding)
Meat fed (5)	0,7,14,21,28	<2
Grain fed (5)	0,7,14,21,28	<2
Enterococcus (5)	0,7,14,21,28	<2
B. frag. ss. frag.* (5)	0	<2
	7,14,21,28	>40
B. frag. ss. frag.* + Enterococcus (20)	0	<2
	7	22
	14	45
	21,28,42	>60
B. frag. ss. frag.* (killed) + B. frag. ss. dist.+ (10)	0	<2
	5	>20
B. frag. ss. frag. (killed)* (5)	0	<2
	5	4
B. frag. ss. vulgatus+ (5)	0,5	<2
B. frag. ss. dist. + (8)	0,7	<2
B. frag. ss. frag.* (no cecal contents or $BaSO_4$) (7)	0	<2
	5	24
Fecal inoculum (6)	0	<2
	7	7

* encapsulated + unencapsulated

This data would indicate that the RABA is useful in detecting antibody to the capsular material in the experimental model; that it is sensitive and can detect antibody as early as 5 days after implantation and also that it is specific.

H. Antibody in Patients with B. fragilis Infection

Paired sera have been obtained from 10 patients with proven B. fragilis infection. Significant changes in antibody levels were demonstrated

in all three patients with subspecies fragilis infection, but in only 3 of 7 patients with infection involving other subspecies. It is of interest that two of these three infections involved more than one subspecies of organisms. Organisms of subspecies fragilis are identified by negative sugar fermentations and negative indole reaction. It is possible therefore in mixed cultures to "mask" the presence of this subspecies by another subspecies fermenting an appropriate sugar or producing an indole reaction. Also, it is possible that the capsular antigen is shared by strains of other subspecies. However, a most intriguing possibility is that mixed infection with subspecies other than fragilis induces antigen formation. This possibility is also suggested by our finding that rats infected with a fecal inoculum, not containing organisms of ss. fragilis, developed antibody rises to the capsular substance.

II. Endotoxin

Endotoxins of aerobic gram-negative bacteria have been studied extensively for many years. However, the lipopolysaccharides of anaerobic gram-negative rods have not been subjected to a similarly intense investigation until relatively recently.

Using aqueous phenol extracts of Bacteroides fragilis fragilis, Hofstad and Kristofferson (23) found that the lipopolysaccharides of this organism lacked two essential sugars of classical endotoxins: 2-keto-3-desoxyoctonate (KDO), and heptose. They also found that this preparation had relatively weak endotoxin potency. When the lipopolysaccharide was administered in relatively high doses (200 ug per rabbit), however, it induced a local Schwartzman reaction. Using a similar extraction procedure Sonnenwirth, et al. (24) found KDO in the endotoxin of B. fragilis fragilis; endotoxin was detected in the sera of patients with bacteremia due to B. fragilis by means of the Limulus lysate assay, another indication of the presence of a lipopolysaccharide.

Endotoxins of gram-negative bacteria are found uniformly associated with the outer membrane complex of antigens. In our studies of theouter cell wall membrane of B. fragilis, the membrane was found to contain an important protein component which was shared by all studied strains of subspecies fragilis, but which was not shared by representative strains of other subspecies (1). The outer wall membrane contained two major antigenic fractions which were separable by the disaggregation of one of these components by chromatography on a Sephadex G-100 column (Pharmacia, Uppsala, Sweden) in an endotoxin-disaggregating buffer. The disaggregated antigenic fraction contained lipid and carbohydrate; another carbohydrate portion which has been shown to be a capsular polysaccharide (25) eluted at the void volume of the column, and contained four of the five sugars found in the disaggregated antigenic fraction. The void dolume fraction had some protein in addition to the carbohydrate. Each fraction had one sugar that was not found in the other fraction. Both antigenic fractions contained lipid. However, the disaggregation of the non-protein fraction in the endotoxin-disaggregating buffer led us to assume that there existed a more

intimate relationship between lipid and carbohydrate in this fraction. Both fractions contained hexose, hexosamine, and methyl pentose in different molar ratios. Both fractions lacked KDO and heptose, which form part of the inner core of bacterial endotoxins.

We have now compared B. fragilis outer membrane lipopolysaccharide with lipopolysaccharide extracted from cells with aqueous phenol, and find the two preparations to be chemically and biologically similar. Both preparations lack many of the biologic characteristics of endotoxins.

Purification of the outer membrane. (1,26) Pelleted organisms were suspended at 24 C in a buffer containing 0.05 M sodium phosphate, 0.15 M NaCl, and 0.01 M ethylene-diaminetetraacetic acid (EDTA), adjusted to pH 7.4. The final volume was approximately 1/50th that of the original culture. The suspension of organisms was incubated at 60 C for 30 min, subjected to mild shearing by passage through a 25-guage hypodermic needle by manual pressure, and mixed for 10 sec in a Waring blender.

Organisms were pelleted from the suspension by centrifugation at 12,000 x g at 4 C for 20 min; the supernatant fluid was then centrifuged (to pellet the membranes) at 80,000 x g at 4 C for 2 h. Both centrifugations were repeated and the final product, a clear gel-like pellet, was stored frozen in water.

Separation of lipopolysaccharide from the outer membrane complex. (1) Loosely bound lipid, when specified, was removed from the complex after precipitation of the outer membrane from 0.12 N NaCl with 80% ethanol (vol/vol); the precipitate was washed twice with chloroform-methanol (2:1 vol/vol). After centrifugation at 16,000 x g at 4 C and reextraction of the pellet with chloroform-methanol, the two supernatant fluids were pooled and dried by evaporation under a stream of nitrogen. The material which was insoluble in chloroform-methanol was dried and then solubilized in 5 cc of the endotoxin-disaggregating buffer (NaD buffer) which contained 0.05 M glycine, 0.01 M EDTA, and 0.5% sodium desoxycholate, adjusted yo pH 9.0 with 6 N NaOH. This material was then chromatographed on a 1.6- x 82-cm Sephadex G-100 column equilibrated in NaD buffer, and 2-ml fractions were collected. In some experiments, for a definition of the elution profile of the preparations, outer membranes were intrinsically labeled with (H^3) glucose, leucine, or acetate. Two peaks of radioactivity were noted (1). No further disaggregation was found when the buffer was prepared with 3% sodium desoxycholate.

The void volume material (peak 1, $>10^5$ daltons) was collected, pooled and concentrated to 5 ml by means of an ultrafiltration cell with a PM-30 membrane (Amicon Corp., Lexington, Mass.). The remainder of the material (peak 2), with an estimated molecular size of 12,000 daltons, was recovered and treated in the same way, except that it was concentrated on a UM-2 membrane (Amicon) because of the smaller size of the material. Both concentrates were precipitated with four volumes of cold absolute ethanol. For removal of the remaining sodium desoxycholate, the pellet was washed twice with 80% ethanol; in some experiments the pellet was also washed twice with absolute alcohol to remove the remaining lipid (8). In other experiments, after the peak 2 material was collected and concentrated, the pool was resuspended in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.3, and rechromatographed on a 2.5 x 90 cm Sepharose 4B column equilibrated in this buffer.

This procedure was done to demonstrate repolymerization of the peak 2 material.

Other methods of endotoxin preparation. Endotoxin was also prepared by means of a modification of the phenol/water method of Westphal (2). Pelleted whole bacteria were suspended to make a concentration of 20mg/ml (wet weight in water). Eleven volumes of 88% hot (68 C) phenol were added to nine volumes of bacterial suspension and the mixture was vigorously shaken for 5 min at 68 C. The two phases were separated in a separatory funnel at 4 C for 18 h. After removal of the aqueous phase, the extraction of the phenol phase was repeated with nine volumes of water. The next day, the aqueous phases were combined and the excess phenol was removed by triple extraction with ether. Excess ether was removed from the aqueous extract in a separatory funnel; a stream of air was then blown across this phase, and it was then concentrated 10 times on a PM-30 membrane (Amicon). The concentrate was precipitated with four volumes of cold absolute alcohol and centrifuged at 10,000 x g for 15 min. The pellet was suspended in neutral phosphate buffered saline and then double treated with DNase, RNase, and trypsin. The clear supernatant was then chromatographed on a 2.5 x 90 cm Sepharose 4B (Pharmacia) column equilibrated in 0.05 M Tris buffer (pH 7.3). The void volume material was collected, concentrated 20 times on a PM-30 membrane, and precipitated from the buffer with four volumes of cold absolute alcohol. This precipitate was suspended in water and lyophilized. The dry material was the aqueous phenol extract.

A similar extraction procedure was done for the EDTA treated organisms which were separated by centrifugation from the outer membrane preparation in the supernatant. This was done because electron micrographs of organisms (taken after separation of the outer membrane) have shown that a majority of bacteria still contain intact outer membranes (1). A similar extraction was done on EDTA treated organisms that were not sheared. Additionally, the phenol-chloroform-petroleum ether extraction for rough mutants was done as described by Galanos et al. (27). Aqueous phenol extracted endotoxin from Escherichia coli 0:127 and Salmonella typhi 0:901 were kindly supplied by Dr. William R. McCabe.

Analytical methods. Fatty acid ester and amide content was determined by the procedure of Snyder and Stevens (28), as modified by Haskins (29). Total lipid content was estimated by the method of Bligh and Dyer (30). 2-keto-3-deoxyoctonate content was estimated by the method of Weissbach and Hurwitz (31) as modified by Osborne (32). Total heptose content was determined by the cysteine-H_2SO_4 method of Osborne (32). Papers for chromatographic analysis of carbohydrates were run in a solution of ethyl acetate:pyridine: water (120:50:40) for 10 h after hydrolysis of 200 µg of samples in 2 N HCl for 4 h at 100 C. Papers were developed by means of silver nitrate and NaOH (33).

Tests for biologic activity. The Limulus lysate assay was kindly done by Dr. William R. McCabe, according to the method of Levin (34).

Tests for toxicity were done on chicken embryos as described by Smith and Thomas (35). Serial 10-fold dilutions of sample in 0.1 ml sterile 0.15 M saline were injected into lowered chorioallantoic membrane of 10-day old

chicken embryos. Six to 10 eggs received injections of each concentration. After the injection port was closed with tape, the eggs were incubated in a non-turning tray at 34 C ± 0.5 C for 24 h. At this time the number of deaths was determined and 50% lethal end points were calculated by the method of Reed and Muench (36). Control infections of sterile saline and of an endotoxin of group C Neisseria meningitidis were performed. This endotoxin was prepared by means of the NaD buffer technique (1,37).

Skin sites for testing of the dermal Shwartzman reaction were prepared in groups of New Zealand white rabbits, 1.0 - 1.5 kg and between 2 and 4 months old, by the intradermal injection of serial dilutions of 0.25-ml samples of test material. The reaction was provoked 21 h later by the intravenous injection of 0.5-ml samples. Hemorrhage or necrosis of the skin within 6 h after the provocative dose was given was recorded as a positive reaction. The method of Reed and Muench (36) was again used to calculate 50% end-points.

Five extraction techniques were used to compare qualitative chemical and biological activities of the endotoxin of B. fragilis. Essentially no extractable antigen was obtained from 5 g (dry weight) of the organisms by means of the phenol-chloroform-petroleum ether method (27), a finding that indicates that firmly bound lipids were not a major component of this endotoxin.

Paper chromatograms of the hydrolyzates of the four other preparations (stained for sugars) are seen in figure 9. Material from peaks 1 and 2 resulting from the chromatography of the outer membrane extract on a G-100 column have four identical sugars shared by both fractions (a-d), but each fraction has a fifth sugar not found in the other (e and f). The aqueous phenol extract contains sugars similar to those found in the peak 2 material (a-e), as does the EDTA treated phenol extract which was not sheared. The aqueous phenol extract from the EDTA- and shear-treated organisms contain both of the extra sugars (e and f). These results substantiate that the peak 2 material is similar to the aqueous phenol extract from this organism. In another portion we have shown that the peak 1 material is a capsular polysaccharide.

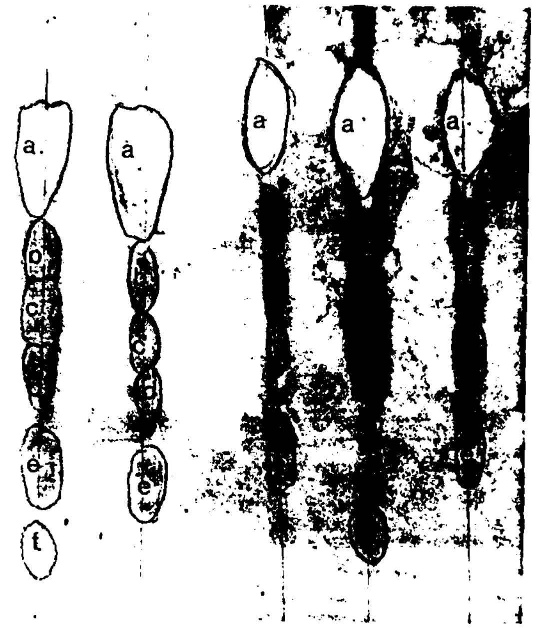


Figure 9. Paper chromatogram of various antigen preparations stained for sugars. Left to right: 1) EDTA-shear-phenol extract 2) classic phenol/ water extract 3) EDTA-non-sheared-phenol extract 4) Peak 1 extract 5) Peak 2 extract.

Table 9 demonstrated the loose association of polysaccharide and lipid of peaks 1 and 2. Non-lipid extracted outer membrane was solubilized in NaD buffer, and chromatographed on G-100 yielding peaks 1 and 2. Twenty-four percent of the dry weight of peak 1 and 46% of the dry weight of peak 2 was lipid. The intact membrane contained 1.7 μEq fatty acid ester/mg. When the membrane was twice extracted with chloroform-methanol prior to chromatography and was then chromatographed on Sephadex G-100 in NaD buffer, 0.42 μEq fatty acid ester/mg was found in peak 1 and 0.18 μEq fatty acid ester/mg was found in peak 2. By further washing of both peaks with 80% and absolute alcohol, the fatty acid ester content of peak 1 could be reduced to 0.1 μEq/ml and to 0.2 μEq/mg in peak 2.

Table 9

Change in lipid content of outer membrane fractions after successive washings with organic solvents.

Peak	Non-lipid extracted fraction (%)[a]	Chloroform-methanol[b] extracted fraction (uEq/mg)	Chloroform-methanol and alcohol extracted[b] fraction (uEq/mg)
1	24	0.42	0.1
2	46	0.18	0.2

[a] Figures represent total lipid content (percentage of dry weight).

[b] Figures represent fatty acid ester content (expressed as uEq fatty acid per mg antigen).

So that the hypothesis that peak 2 was a disaggregated lipopolysaccharide could be tested, this extract was rechromatographed on Sepharose 4B equilibrated in Tris (figure 10).

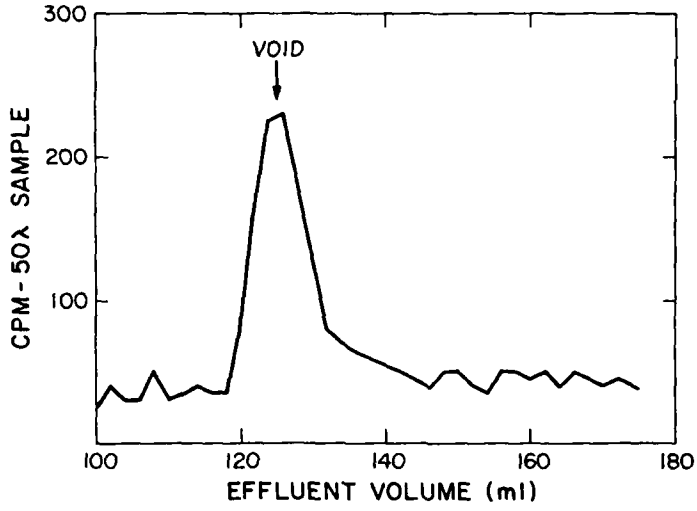


Figure 10. Elution profile of (H^3) labeled peak 2 demonstrating reaggregation in absence of NaD buffer. Identical elution volume was noted with peak 2 material after washing with chloroform-methanol or chloroform-methanol and absolute alcohol. CPM=counts per minute of 50 λ sample.

Three preparations of peak 2 were chromatographed separately; the intact peak (no lipid extracted), the chloroform-methanol extracted peak 2; and the chloroform-methanol and alcohol extracted peak 2 (table 9). All three preparations eluted at the void volume of the Sepharose column, a finding that indicates partial sizes greater than 5×10^6 daltons. Therefore, these preparations all aggregated in the absence of NaD buffer, and might again be disaggregated in NaD buffer (figure 11); this possibility was tested by repeated chromatography on Sephadex G-100 in NaD buffer with elution at a molecular size of 12,000 daltons.

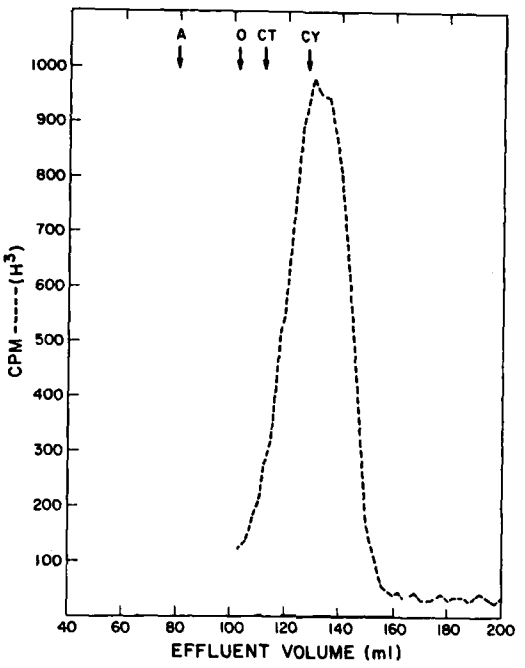


Figure 11. Elution profile of (H^3) labeled lipopolysaccharide extracts demonstrating disaggregation after chromatography in NaD buffer containing G-100 column. Elution peaks of molecular size markers are indicated as: A = aldalase (void volume) (158,000), O = ovalbumin (45,000), CT - chymotrypsinogen A (25,000), CY - cytochrome C (12,700).

Column chromatography of the aqueous phenol extract demonstrates that like the material of peak 2, the extract fully disaggregated in NaD buffer. The EDTA-shear-phenol extract, which contains the sugars found in peaks 1 and 2, disaggregates in the NaD buffer when chromatographed into the two fractions, a finding one would anticipate from the sugar content represented on paper chromatography. This group of experiments demonstrates that there is a physical-chemical difference between material in peaks 1 and 2 other than the additional sugar we have noted. The difference seems to be related to a greater affinity of polysaccharide for lipid in the peak 2 material, because the lipid portion of the lipopolysaccharide is responsible for disaggregation in NaD buffer.

We next tested our preparations for biological endotoxicity. The results of the Limulus lysate test are shown in table 10. There were no significant differences among the concentrations at which non-lipid extracted peaks 1 and 2, aqueous phenol, EDTA-shear-phenol, EDTA-non-shear-phenol, and intact outer membrane caused gelation of the lysate. There is some suggestion that the lipid extracted peaks 1 and 2 are less potent than their non-lipid extracted duplicates. All preparations are significantly less potent on a concentration basis than either of the control endotoxins.

Table 10

Results of the Limulus lysate test

Preparations	Concentration (γ/ml)[a]
Outer membrane	0.125
Peak 1 (non-lipid extracted)	0.03
Peak 2 (non-lipid extracted)	0.06
Classic phenol	0.06
Phenol-EDTA-shear	0.06
Phenol-EDTA	0.06
Peak 1 (lipid extracted)	0.156
Peak 2 (lipid extracted)	0.290
Escherichia coli 0:127	0.003
Salmonella typhi 0:901	0.003

[a]Figures represent concentrations of individual preparations at which gelation of the lysate occurred.

Non-lipid extracted peak 1, peak 2, outer membrane, lipid component of outer membrane, and aqueous phenol extract were all tested for their ability to kill 10-day old chick embryos when injected into the chorioallantoic membrane. A control endotoxin of group C Neisseria meningitidis prepared in a manner similar to that for the peak 2 extract was tested. The results are shown in table 11. None of the B. fragilis preparations showed lethality when administered in doses of up to 200 µg per egg. However, the control endotoxin (group C Neisseria meningitidis), had an LD_{50} of 1.2 µg per egg.

Table 11

Lethality of various preparations on 10-day old chick embryos after injection into the chorioallantoic membrane

Preparation	LD_{50} (µg/egg)
Outer membrane	>200
Peak 1 (non-lipid extracted)	>200
Peak 2 (non-lipid extracted)	>200
Classic phenol extract	>200
Group C endotoxin, Neisseria meningitidis [a]	1.2

[a] The endotoxin of group C Neisseria meningitidis was prepared in a manner similar to that used for the peak 2 (non-lipid) extract.

Finally, various preparations were tested for their ability to induce the local Shwartzman phenomenon in groups of rabbits. An endotoxin of Salmonella typhi 0:901 extracted by aqueous phenol was used as a control. The

results are shown in table 12. None of the B. fragilis preparations induced the local Shwartzman reaction when given in doses as high as 1 mg. However, the control (S. typhi endotoxin) gave a positive result in all five rabbits tested, with a 50% endpoint of 3 µg. These experiments demonstrate that in our hands, B. fragilis endotoxins have minimal biologic activity in the tests we performed.

(See Table 12, page 34)

So that we could be sure the endotoxin of this organism had not been missed by any of the standard extraction procedures used, whole bacteria were tested for their ability to induce the local Shwartzman phenomenon. Twenty-one hours after graded preparative doses of up to 2,500 µg dry weight of B. fragilis (killed by exposure to ambient atmosphere) were given to groups of five rabbits, provocative doses of 1,000 µg were administered. No reactions were noted in any rabbit, an indication of the absence of biologically active endotoxin. Groups of five rabbits were similarly tested with strain 99M group B Neisseria meningitidis and strain 526 of Pseudomonas aeruginosa. Both organisms were killed by heating at 60 C for 30 min. The 50% endpoint for meningococcus was 600 µg (preparative dose, dry weight) and 900 µg for pseudomonas as determined by the method of Reed and Muench (36). A 1,000 µg provocative dose was also used in these rabbits. The weak endotoxin potency of these whole cells could be anticipated because of their insolubility and the vastly more potent endotoxicity of soluble extracts.

In this portion of the study, we have characterized by several techniques an endotoxin like molecule isolated from Bacteroides fragilis fragilis. This component has been separated from purified preparations of outer membrane by disaggregation via NaD buffer and column chromatography. We have also isolated a chemically similar component by the aqueous phenol technique (2). It is of interest that these extracts lack KDO and heptose, two of the characteristic sugars of aerobic bacterial endotoxins. Using aqueous phenol extracts, Hofstad and Kristofferson also noted that endotoxins of B. fragilis lacked these sugars (23). Sonnenwirth (24), however, found KDO in the endotoxins of some strains of B. fragilis. Differences in strains may cause this divergent data. In classical endotoxins, KDO is linked to lipid A. We have demonstrated that in these preparations, KDO is absent. The character of the linkage between the lipid moiety and the polysaccharide is unknown. However, by simple washing with chloroform-methanol and alcohol, the greater part of the lipid is removable. This molecule, washed in organic solvent, still retains some lipid which allows repeated disaggregation in NaD buffer and reaggregation in the absence of this buffer. Therefore, we feel there is a close association between lipid and polysaccharide.

These studies have shown that the lipopolysaccharide of Bacteroides fragilis fragilis, in our hands, lacks some characteristic biologic functions of an endotoxin. This lipopolysaccharide gels the Limulus lysate at higher concentrations than do the aerobic endotoxins. This finding is in agreement with those of Sonnenwirth who has demonstrated a positive Limulus test in the sera of patients with sepsis due to Bacteroides. We were unable to

Table 12. Comparison of potencies of various endotoxin preparations in effecting the local Shwartzman phenomenon in groups of rabbits.

Dose (µg)		Endotoxin preparation [a]						
Preparative	Provocative	Outer membrane	Peak 1	Peak 2 (non-lipid extract)	Peak 2 (lipid Extract)	EDTA-shear phenol	Phenol	Salmonella typhi O:901
1,000	500	0/5	-	-	0/5	0/5	-	-
900	400	-	0/5	-	-	-	-	-
600	300	-	-	0/4	-	-	0/2	-
500	500	0/5	-	-	0/5	0/5	-	-
250	500	0/5	-	-	0/5	-	-	-
100	500	0/5	-	-	0/5	-	-	-
50	500	0/5	-	-	0/5	-	-	-
50	10	-	-	-	-	-	-	5/5
25	10	-	-	-	-	-	-	4/5
5	10	-	-	-	-	-	-	3/5
2.5	10	-	-	-	-	-	-	2/5
0.5	10	-	-	-	-	-	-	1/5

[a] Figures represent number of rabbits with positive Shwartzman reaction/number in group tested. (-) = no test performed.

demonstrate lethality in chick embryos or to induce the local Shwartzman reaction in rabbits even with the use of very large quantities of material. These tests were done with and without the lipid having been removed from the preparations. The non-lipid extracted molecules are soluble and lack endotoxicity; therefore, an insoluble lipid A reported to lack endotoxicity is not the cause of this lack of biologic function. Despite the presence of 46% lipid in peak 2, we have shown that this lipid is largely loosely bound. As one might predict with so little tightly bound lipid present in this molecule, we obtained no lipopolysaccharide with use of the phenol-chloroform-petroleum ether method of extraction of rough mutant endotoxins (27). One may consider a spectrum of types of lipopolysaccharide, each type consisting of different relative amounts of lipid and polysaccharide. It would seem the moieties of Bacteroides fragilis fragilis represent lipopoly-saccharides at one extreme of this spectrum, containing much polysaccharide and small amounts of tightly bound lipid. The lipopolysaccharide of R mutants, on the other hand, represents the other extreme of the spectrum, containing much lipid and small amounts of polysaccharide.

The lack of local Schwartzman phenomena after injection of whole bacteria into rabbits has also assured that endotoxin was probably not missed by these standard extraction methods.

This lipopolysaccharide with limited or no endotoxic properties may account for the apparently low incidence of disseminated intravascular coagulation in patients with sepsis due to Bacteroides fragilis, a theory which has been hypothesized previously (38).

III. Bacteroides melaninogenicus

We have now begun similar immunochemical investigations of Bacteroides melaninogenicus. This organism is the predominant species of Bacteroides isolated from lung abscesses.

B. melaninogenicus is thought to be responsible, in part, for periodontal disease and has been shown, in fact, to be essential in certain synergistic anaerobic infections of the mouth (i.e., the bacterial mixture, in the absence of B. melaninogenicus, is non-infective). Very little is known about the antigenic make-up and biochemical structure of B. melaninogenicus and consequently about virulence factors and the immune response to infection by B. melaninogenicus. Methods have been developed in this laboratory in the study of B. fragilis that are useful in the study of B. melaninogenicus.

The outer cell membrane may play a role in the virulence of Bacteroides melaninogenicus, as it does in many other bacterial species, and is very likely the site of antigenic recognition by the host. Purification of the cell membrane by others has been notably incomplete to date, and identification of purified antigens is almost non-existent. Therefore, we have begun to study the outer membrane of B. melaninogenicus as follows: first, by isolating and characterizing components of the outer membrane complex; second, by identifying the antigenic components of the outer membrane complex.

Methods and Results

B. melaninogenicus, strain 382 was obtained from the Forsythe Dental Laboratory. Strain 536 was obtained from A.K. Daly, Boston City Hospital Microbiology Laboratory. The bacteria were grown and harvested in 6 liter batches using culture medium described by Sokransky. The isolation procedure used was that described by us previously and included differential centrifugation, EDTA treatment, shearing and ultracentrifugation. The outer membrane complex was then separated on a Sephadex G-100 column using 0.5% Na deoxycholate buffer (endotoxin disaggregating). The eluent demonstrated a major peak at the void volume that consisted of large protein and carbohydrate components (see percentages below) and a smaller second peak (1/3 of total) which contained a majority of carbohydrate and very little protein. The peaks were concentrated with Amicon apparatus, using PM-30 and UM-2 membranes for peaks 1 and 2, respectively.

Peak 1 was found to contain 50% protein and 50% carbohydrate. The carbohydrate portion was composed of hexose, methyl pentose and hexosamine (6:2:1). Peak 2 contained no protein and was composed of hexose, methyl pentose and hexosamine (9:2:1). SDS-polyacrylamide gel electrophoresis showed several distinct bands in peak 1 and none in peak 2, when stained for proteins.

Antiserum was developed in New Zealand white rabbits against live organisms of strain 382. 536 antiserum had previously been raised. Ouchterlony double diffusion in agar was done using antisera from strains 536 and 382 as well as pre-immunization serum with the following antigens: 382-peak 1, 382-peak 2, whole membrane-536. The results were as follows: there was no precipitin line with 536 and anti-382 (subspeciation has not yet been done so subspecies or strain specificity has not yet been determined); there were two precipitin lines with whole membrane 536 and anti-536; no lines were seen with any antigen and serum prior to immunization; two lines were seen with peak 1 382 and anti-382; no lines were seen with peak 2 382 and anti-382 (this may well reflect a low concentration of antigen used in the test). Immunoelectrophoresis of peaks 1 and 2 (strain 382) with anti-382 is currently being performed. Separation of the carbohydrate from the protein in peak 1 is currently being performed in order to study further the antigenic structure.

References

1. Kasper, D.L. and Seiler, M.W. 1975. Immunochemical characterization of the outer membrane complex of Bacteroides fragilis subspecies fragilis. J. Infect. Dis. 132:440-450, 1975.

2. Westphal, O., Luderlitz, O. and Bister, F. 1952. Über die extraktion von bakterien mit phenol/wasser. Z. Naturforsch. 7B:148-155.

3. Luft, J.H. 1961. Improvements in epoxy resin embedding methods. J. Cell Biol. 9:409-417.

4. Luft, J.H. 1966. Fine structure of capillary and endocapillary layer as revealed by ruthenium red. Fed. Proc. 25:1773-1778.

5. Farr, R.S. 1958. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. Infect. Dis. 103:239-262.

6. Brandt, B.L., Wyle, F.A. and Artenstein, M.S. 1972. A radioactive antigen binding assay for Neisseria meningitidis polysaccharide antibody. J. Immunol. 108:913-920.

7. Roe, J.H. 1955. The determination of sugar in blood and spinal fluid with the anthrone reagent. J. Biol. Chem. 212:335-343.

8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

9. Danielsson, D., Lambe, D.W., Jr. and Persson, S. 1972. The immune response in a patient to an infection with Bacteroides fragilis fragilis and Clostridium difficile. Acta. Pathol. Microbiol. Scand. (Sec. B) 80:709-712.

10. Rowley, D.J. 1971. Endotoxins and bacterial virulence. J. Infect. Dis. 123:317-327.

11. MacLeod, C.M. and M.R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. J. Exptl. Med. 92:1-9.

12. Casciato, D.A., Rosenblatt, J.E. Goldberg, L.S. and R. Bluestone. 1975. In vitro interaction of Bacteroides fragilis with polymorphonuclear leukocytes and serum factors. Infec. Immun. 11:337-342.

13. Muschel, L. 1965. Immune bactericidal and bacteriolytic reactions. In G.E.W. Wolstenholme and J. Knight (ed.) Ciba Foundation Symposium on Complement. Little, Brown. Boston, p. 155.

14. Aranki, A., Freter, R. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. Am. J. Clin. Nutr. 25:1329.

15. Onderdonk, A.B., Weinstein, W.M., Sullivan, N.M., Bartlett, J.G. and Gorbach, S.L. 1974. Experimental intraabdominal abscesses in rats: Quantitative bacteriology of infected animals. Infect. Immun. 10:1256.

16. Moore, W.E.C. and Holdeman, L.V. 1974. Human fecal flora; the normal flora of 20 Japanese-Hawaiian. Appl. Microbiol. 27:961.

17. Anaerobe Laboratory Manual. Edited by L.V. Holdeman, W.E.C. Moore. Blacksburg. Virginia Polytechnic Institute and State University Anaerobe Laboratory, 1972.

18. Edwards, P.R. and Ewing, W.H. 1972. Identification of Enterobacteriaceae. 3rd Edition, Burgess Pub. Co., Minneapolis, Minn.

19. Weinstein, W.M., Onderdonk, A.B., Bartlett, J.G., et al. (in press, 1976) Experimental intraabdominal abscesses in rats: Development of an experimental model.

20. Altemeier, W.A., Culbertson, W.R., Fullen, W.D., et al. 1973. Intra-abdominal abscesses. Amer. J. Surg. 125:70.

21. Gorbach, S.L. (In press, 1976) Treatment of intraabdominal sepsis. Ann. Int. Med.

22. Gorbach, S.L. 1971. Intestinal microflora. Gastroenterol. 60:1110.

23. Hofstad, T. and Kristofferson, T. 1970. Chemical characteristics of endotoxin from Bacteroides fragilis NCTS9343. J. Gen. Microbiol. 61:15.

24. Sonnenwirth, A.C., Yin, E.T., Sarmiento, E.M. and Wessler, S. 1972. Bacteroidaceae endotoxin detection by Limulus assay. Am. J. Clin. Nutr. 25:1452-1454.

25. Kasper, D.L. (in press, 1976) The polysaccharide capsule of Bacteroides fragilis subsepcies fragilis: morphological and immunochemical definition. J. Infect. Dis.

26. Zollinger, W.D., Kasper, D.L., Veltri, B.S. and Artenstein, M.S. 1972. Isolation and characterization of a native cell wall complex from Neisseria meningitidis. Infect. Immun. 6:835-851.

27. Gelanos, C., Luderitz, O. and Westphal, O. 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. 9:245.

28. Snyder, F. and Stephens, N. 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochim. Biophys. Acta 34:244-245.

29. Haskins, W.T. 1961. Spectrophotometric determination of fatty acid amides in lipids. Anal. Chem. 33:1445-1446.

30. Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction

and purification. Can. J. Biochem. Physiol. 37:911-917.

31. Weissbach, A. and Hurwitz, J. 1959. The formation of 2-keto-3-deoxy-heptonic acid in extracts of Escherichia coli B. J. Biol. Chem. 234: 705-709.

32. Osborne, M.J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto, 3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium. Proc. Nat. Acad. Sci. USA 50:499-506.

33. Smith, I. 1960. Sugars. In I. Smith (ed.), Chromatographic and electrophoretic techniques, p. 246-260. William Heineman Medical Books, Ltd., London, and Interscience Publishers, New York.

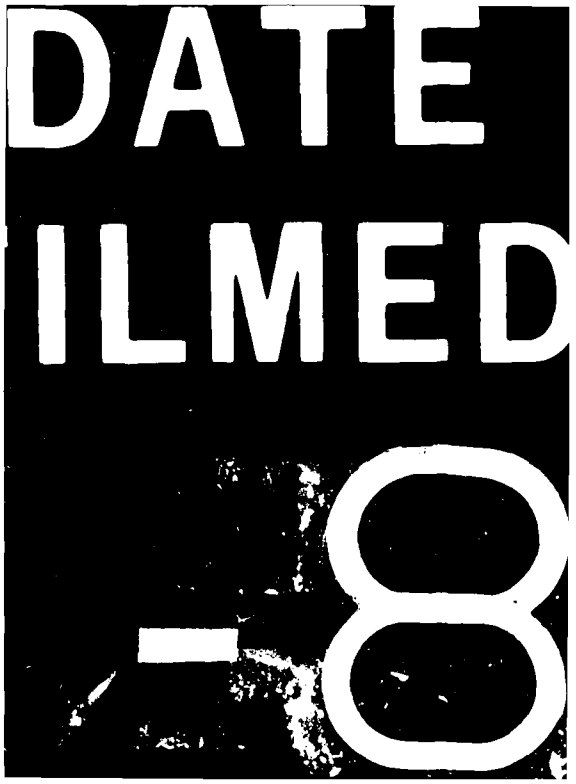
34. Levin, J., Poore, T.E., Zauber, N.P. and Oser, R.S. 1970. Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. N. Engl. J. Med. 283:1313.

35. Smith, R.T. and Thomas, L. 1956. The lethal effects of endotoxins on the chick embryo. J. Exp. Med. 104:217-231.

36. Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.

37. Sadoff, J.C. and Artenstein, M.S. 1974. The outer cell wall membrane of Pseudomonas aeruginosa. J. Infect. Dis. 130S (Suppl.):S81-S90.

38. Yoshikawa, T.T., Chow, A.W. and Guze, L.C. 1974. Bacteroidaceae bacteremia with disseminated intravascular coagulation. Am. J. Med. 56:725-728.


DATE
ILMED
-8